RESEARCH REPORT
SWINE HEALTH

Title: Subunit Vaccines to Prevent and Control PRRS Viral Infections
NPB #97-1979

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Abstract:
We have chosen the well-characterized North American Prototype Strain of PRRSV, VR-2332 for our studies. We have completed the construction of the mWAP-transgene and we have successfully produced 17 mice, which carry the mWAP-ORF5 transgene. Most female founders have been outbred with wild type mice and we have collected milk samples for further analysis. Our data show that the mWAP-ORF5 gene was stable integrated into the genome at a frequency of 10% and transmitted to the offsprings at a frequency of 50%. However, analysis of the milk samples collected from transgenic mice during lactation failed to show a positive response with polyclonal PRRSV antiserum. But mRNA isolated from the mammary gland biopsies during lactation indicated the accumulation of the ORF5 message in the mammary tissue. Our analysis was further complicated by the unavailability of PRRSV antiserum with good IFA titers and good SVN activity. Thus I have not been able to proceed with the remainder of project objectives. In order to address the problem scientifically, I have obtained support from other sources to continue this investigation. We are currently seeking to make GST-ORF5 fusion protein that will be used to generate PRRSV-ORF5 specific serum. I will make every attempt to share results with the NPPC staff and I am very grateful for their support of this project.

Objectives: The hypotheses of this study is that transgenic mice carrying the ORF-5 cDNA of PRRSV under the control of mWAP gene will produce recombinant E1 (rE1), ORF-5 encoded protein of PRRSV in the milk during lactation. Purified rE1 will elicit a protective immune response in pathogen-free pigs and will be an effective antigen in ELISA and blocking assays. The short gestation period of mice provides a quick time frame for the generation and analysis of founder transgenic mice and, a facile format to validate the project objectives. The following specific objectives are outlined to validate our hypotheses within the one-year time frame of this proposal
Objective 1. Produce viral proteins (E1) associated with PRRSV in transgenic mice
Objective 2a. Ability of rE1 to elicit protective immune-response

Objective 2b. Ability of rE1 to effectively react with field sera in ELISA assays.

Objective 1:

Construction of mWAP-ORF5 hybrid transgene. A clone of ORF5 sequence was obtained from a cDNA copy of the mRNA genome encoding ORF 2-7 for isolate VR-2332. The cDNA sequences for a clone of VR-2332 was provided by Dr. K. S. Faaberg (Department of Veterinary-Pathobiology, University of Minnesota). mWAP is tissue specific promoter and contains genetic codes which direct the expression of the foreign protein in the mammary gland during lactation. The WAP clone (pUC Not I+) containing ~4.2 kb of WAP 5' promoter region, and ~1.6 kb of WAP 3' UTR including the flanking 3' end was modified from a 7.2 kb genomic WAP fragment. The cDNA for ORF5 was end modified by PCR to produce Kpn I endonuclease sites located immediately 5' to the ATG start codon and 3' of the stop sequence in the 3' UTR. Oligonucleotide primers were synthesized according to the nucleotide sequence information from VR-2332 and were used to amplify the respective cDNA from pT712 template provided by Dr. M. P. Murtaugh. Primer 1 (5' CGGGGTACCATGTTGGAGAAATGCTTGAC) and primer 2 (3' CGGGGTACCCTAAGGACGACCCCATTG) were specific primers for the ORF5 region. The Kpn I restriction site was incorporated at both the 5' and 3' end of the primers to facilitate cloning. After DNA sequence verification, ORF5 was individually subcloned into the pUC Not I+ expression vector. E. coli strain DH5 was transformed by electroporation and colonies containing the vectors will be isolated and grown. Plasmids was isolated from clones using alkaline lysis and constructs excised by Not I endonuclease digestion of Not I sites flanking the 5' and 3' ends of each construct. Constructs were purified by low melting point agarose gel electrophoresis, treated with agarase, extracted with chloroform/phenol and precipitated with 0.6 volume isopropanol. Pellets were suspended in 10 mM Tris-HCl and 1 mM EDTA pH 7.2 and the hybrid DNA construct was submitted for microinjection.

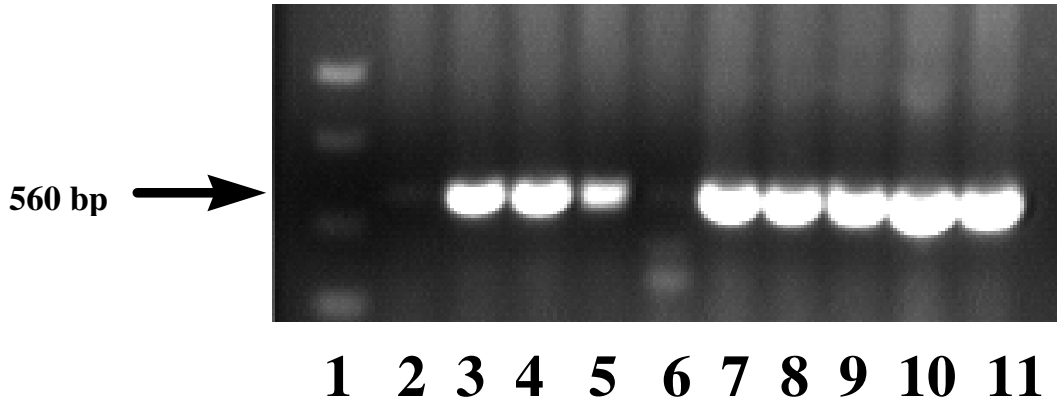
Generation of Transgenic Mice. Transgenic mice were produced by the standard techniques of pronuclear injection at the microinjection facility at the University of Minnesota. mWAP-ORF transgene was microinjected into single celled embryos obtained from female CD1 mice and the microinjected embryos will be reimplanted into a surrogate mother. The pregnancy was allowed to proceed to full term (five weeks) and offspring mice were analyzed for the transgene by analyzing the tail snips for the foreign DNA. In brief, mice resulting from microinjection were screened by PCR using primers that bracket the junction of mWAP and VP-cDNA sequences (5'→ 3'). Tail tissue was biopsied from pups that are approximately 20 days old and DNA was isolated from the tail tissue by a standard procedure outlined in reference. Transgenic founder mice were identified by polymerase chain reaction (PCR) using ORF5 specific primers. Mammary tissue biopsies was obtained during lactation from a female founder and ORF-mRNA levels will be determined by Northern analysis. Milk samples are being collected from lactating females under anesthesia and will be used for subsequent analysis. Transgenic mice are lactating normally and have no phenotypic abnormalities.

RESULTS

Production of transgenic mice. A total of 65 mice were born from 12 recipients that had received zygotes microinjected with mWAP-ORF5 transgene. Initial screening of founder animals by PCR analysis of tail DNA indicated that 17 mice contained the construct, resulting in an integration frequency of 26%. **Figure 1** demonstrates the detection of mWAP-ORF5 transgene in select mouse tail DNA by PCR: lane 1, 123 bp ladder; lane 2, control mouse DNA; lane 3, 75 copy standard; lane 4, 38 copy standard; lane 5, 7.5 copy standard; lane 6, no DNA; lane 7, founder #1; lane 8, founder #2; lane

9, founder #3; lane 10, founder #4; lane 11, founder #5. All transgenic mice appear normal.

Figure 1: Detection of mWAP-ORF5 transgene in mouse tail DNA by PCR



Two µl of template DNA solution (300 ng/µl) was combined with 23 µl of PCR reaction cocktail (1X PCR buffer, 2 mM MgCl2, 0.2 mM dNTPs, 0.6 µM oligonucleotide primers and 0.625 units Taq polymerase (Promega)] for a total reaction volume of 25 µl. The PCR reactions were subjected after pretreatment to denaturation at 94° C for 45 sec, annealing 55° C for 30 secs, and elongation at 72° C for 45 sec, for 35 cycles. Products were run concurrently with molecular weight standards (123 bp ladder, Sigma Chemical Co.) on a 1% agarose gel stained with ethidium bromide. Positive controls are comprised of 30 ng/ml control mouse DNA mixed with appropriate copy number of construct. We are also able to better assess the translational and transcriptional fidelity of the transgenes, we have outbred the transgenic founders with wild type mice. All pregnancies were allowed to proceed to term and the tail biopsies from resulting offsprings were assayed for the presence of transgene by PCR. Our data indicate that the female founders carrying the mWAP-ORF5 transgene transmitted the gene at 50% efficiency, 8 positive offsprings from a total litter size of 16 (see figure 2, select samples illustrated). All the transgenic female mice appear to be normal phenotypes, fertile with normal litter sizes and lactation. All transgenic males were also fertile.

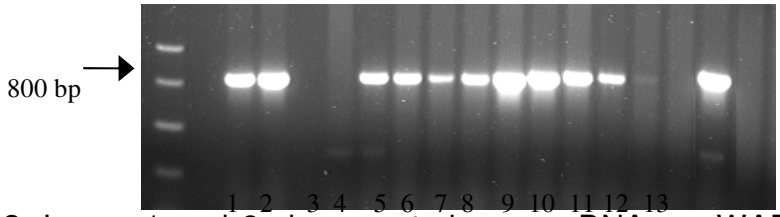


Figure 2: Lanes 1 and 2 show control mouse DNA + mWAP-ORF5 plasmid, lanes 3 and 4 have no DNA, lane 5 shows a mWAP-ORF5 founder #1, lanes 6-13 show offsprings derived from founder #1.

Downstream separation. Milk was defatted with EDTA and centrifuged at 10,000 x g to remove the lipid and the fat phase. The whey phase will be analyzed by polyacrylamide gel electrophoresis under reduced conditions (74) and the presence of the recombinant antigens will be verified by Western blot analysis using PRRSV-specific serum (75). Lysates from PRRSV infected CL2621 cell lines will be used as a positive reference standard for PRRSV proteins in Western blot analysis.

Western Blot analysis. Briefly, after electrophoresis, proteins will be electrophoretically transferred to nitrocellulose sheets, using 25mM Tris, 192 mM glycine, 20% Methanol pH 8.3 as the transfer buffer. The nitrocellulose membrane will be then incubated with

a blocking solution of 0.5% casein solution in 0.01 M Tris-HCl, 0.05 M NaCl, pH 7.5 (TBS), followed by incubation with 1:500th diluted PRRSV specific anti-serum. Finally, the nitrocellulose membrane will be incubated with 1:1000th diluted HRP-conjugated rabbit anti-pig IgG (Sigma) and developed with DAB substrate from Pierce Chemical Company.

Results: Our analyses of the milk samples were complicated by the fact that we were unable to procure PRRSV antiserum that had good reactivity with the standard lysate. To that extent we tested serum provided by Dr. Eric Nelson and Dr. Serge Dea and we were not very successful is getting a good reaction on Western blot. The absence of a monoclonal antiserum against the PRRSV ORF5 made analysis very tedious. However, Dr. Sagar Goyal provided me with serum that had good reactivity and we were not able to see ant recombinant ORF5 expression in the milk. These sera have low IFA and SVN titers. In order to understand this situation in the light of very high copy number gene expression we obtained mammary tissue biopsies were obtained from the mWAP-ORF5 female founders during lactation and mRNA was purified according to the published literature (Biotechniques.1990.8;148-149). Results from Northern analysis of mRNA isolated from the mammary gland biopsies suggest that in two founder mice (founder #1 and #2) the ORF5 mRNA had accumulated in large quantity (see Figure 3). Very little or no ORF mRNA signal was observed in two founder lines studied (founder #3 and #4).

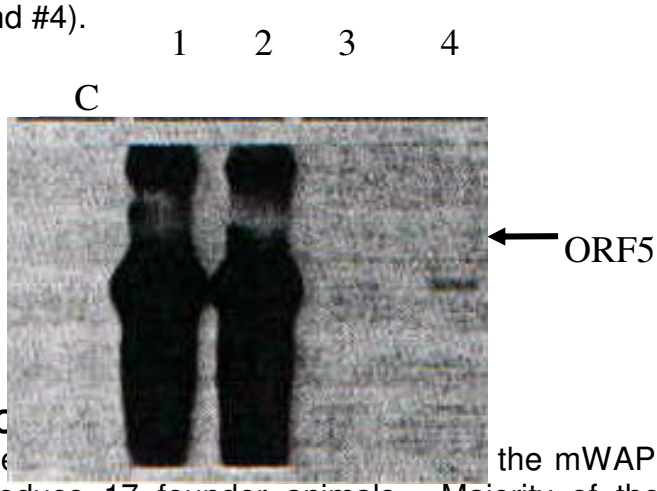


Figure 3 shows the northern blot analysis of gene expression in nontransgenic control mice {C} or transgenic founders {female mice) for the **mWAP-ORF5** transgene. Blots were sequentially hybridized using the ORF5 cDNA probe.

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We the mWAP-ORF5 transgene and we were able to produce 17 founder animals. Majority of these mice transmitted the gene to the offsprings indicating gene integration. Transgenic females had normal litter sizes and provided milk samples without an incident. We have in our freezer about 30-40 ml of milk samples collected from transgenic mice (founders and offsprings) positive for the ORF5 gene. I have obtained funds from additional sources to carry out the following studies: (1) generate PRRSV ORF5 fusion protein (GST-ORF5), (2) generate GST-ORF5 specific serum, (3) validate the serum with positive lysates and (4) re-probe the milk samples with a reactive serum. As an alternative solution, I have sought to reengineer the transgene to include casein signal peptide sequences in the mWAP-ORF5 transgene. I cannot perform animal immunization studies till I confirm the level of expression of recombinant E1 in the milk samples. I anticipate addressing that specific issue in the fall. I will make data available to NPPC as and when it becomes available.